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Award Number: DAMD17-98-1-8047

TITLE: Wnt-1 Signaling in Mammary Carcinogenesis

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REPORT DATE: April 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2000	Annual Summary (16 Mar 99 - 15 Mar 00)

4. TITLE AND SUBTITLE
Wnt-1 Signaling in Mammary Carcinogenesis

5. FUNDING NUMBERS
DAMD17-98-1-8047

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

This report contains colored photographs

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

wnt genes encode a large family of secreted signaling molecules essential for development and oncogenesis. wnt-1, the founding member of the wnt gene family, was initially identified as an oncogene. Ectopic wnt-1 expression causes mammary tumorigenesis in mice, providing a potential model for human breast cancer. However, the cell surface receptor (or receptors) that mediates Wnt-1 signaling has not been identified, and the molecular and biochemical nature of the Wnt signaling pathway is not fully understood. In a research supported in part by this Career Development Award, I propose experiments combining molecular techniques and the axis duplication assay in the *Xenopus* embryo to answer two critical questions: 1) What is the receptor mediating Wnt-1 oncogenic function? 2) How does the Dishevelled protein, which is an essential Wnt signaling component, transduce Wnt-1 signal? Here I provide a progress report on our studies on a potential receptor function of LRP6, a member of the LDL receptor-related protein family, in Wnt signal transduction.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Wnt-1, Frizzled, LRP, Mammary Tumors, Xenopus	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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L. Ue 6-20-00

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Introduction

The Wnt family of secreted signaling molecules plays essential roles in embryogenesis and tumorigenesis (1). The Frizzled (Fz) family of serpentine receptors has been shown to function as Wnt receptors (2-10), but it remains to be elucidated which Fz specifically mediates Wnt-1 oncogenic function and how Fz proteins transduce Wnt signaling.

The Drosophila *arrow* locus defines a novel segment polarity gene whose mutant phenotype resembles that of the *wingless* (Drosophila Wnt-1) mutation (11). *arrow* encodes a transmembrane receptor (11) homologous to two members of the mammalian low-density lipoprotein receptor (LDLR)-related protein (LRP) family, LRP5 and LRP6 (ref. 12-15). Human LRP6 and LRP5 share 71% amino acid identity, and each contains an extracellular domain with multiple LDLR repeats plus EGF (epidermal growth factor) repeats, followed by a transmembrane domain and a cytoplasmic domain lacking any recognizable catalytic motifs (12-15). LRP5 was identified via its location in one of the chromosome loci associated with autoimmune type 1 diabetes mellitus (ref. 13) or as a novel cDNA related to ApoER2 (apolipoprotein E receptor 2) (ref. 14, 15), whereas LRP6 (ref. 12) was isolated by homology to LRP5. A lrp6 gene mutation in mice results in pleiotropic developmental defects, some of which appear to resemble certain Wnt mutant phenotype (16). To study whether/how LRP6 and LRP5 are involved in Wnt signal transduction, including Wnt-1 signaling, we examined the function of LRP6 and LRP5 in Wnt induced secondary axis and neural crest formation in the Xenopus embryo.

Body

1). Ectopic expression of LRP6 activates Wnt signaling

As I outlined in my proposal, activation of the Wnt-1/β-catenin signaling pathway induces dorsal axis formation via activating responsive genes, including nodal-related 3 (Xnr3) and siamois (sia), which are expressed specifically in the Spemann organizer (ref.17). This system provides an excellent model for addressing Wnt signaling mechanism.

Ventral injection of LRP6 RNA into 4-cell stage embryos resulted in dorsal axis duplication in a dose dependent manner (Fig.1a and b). In animal pole explants LRP6 induced Xnr3 and sia expression, but not the expression of brachyury (Xbra) (Fig.1d), which is activated by mesoderm inducers such as activin or bFGF (basic fibroblast growth factor) (17). These results suggest that overexpression of LRP6 specifically activated the Wnt signaling pathway. To further examine whether LRP6 can mediate Wnt signal transduction, RNAs for LRP6 and Xenopus Wnt-5a were co-injected. Wnt-5a was chosen because Wnt-5a alone neither activates the β-catenin pathway nor induces axis formation, but is able to do so in the presence of Frizzled-5 (hFz5), a candidate Wnt-5a receptor (4). While neither Wnt-5a RNA nor a low concentration of LRP6 RNA alone induced axis duplication upon ventral injection, Wnt-5a plus LRP6 synergistically induced axis duplication (Fig. 1a and b) and ectopic Xnr3 expression in the embryo (Fig. 1c), and activated Xnr3 and sia expression in animal pole explants (Fig. 1d). A synergy

was also observed between Fz and LRP6, as hFz5 and LRP6 together strongly induced Xnr3 and sia in the animal pole explant assay (Fig. 1e). Although LRP5 RNA alone did not cause axis duplication, co-injection of RNAs for LRP5 and Wnt-5a did (Fig. 1a), whereas LDLR alone or in combination with Wnt-5a failed to induce axis duplication or Xnr3 and sia expression (Fig. 1a and d). These results suggest that the capability to function in Wnt signaling is specific to LRP5 and LRP6 of the lipoprotein receptor family.

While Wnt-5a/hFz5 can induce complete axis duplication including anterior structures (head) and the notochord (4), Wnt-5A/LRP6 or LRP6 (higher doses) alone induced trunk axis duplication with muscle and neural tissues but lacking head or the notochord (Fig. 1b). It remains unclear whether this is due to quantitative or qualitative differences between Wnt-5a/LRP6 and Wnt-5a/hFz5 co-injections under these experimental conditions.

We also analyzed LRP6 effect on neural crest formation, which is another Wnt-dependent developmental process in vertebrates (18-22). It has been shown that ectopic Wnt expression enhances, whereas lack or inhibition of Wnt signaling inhibits neural crest formation (18-22). While injection of LDLR did not affect neural crest formation, injection of LRP6 into one blastomere at the 2-cell stage significantly expanded neural crest progenitors in the injected half of the embryo, as determined by the expression of slug, a neural crest-specific transcription factor (Fig. 2a and b). Thus, overexpression of LRP6 also mimicked Wnt signaling during neural crest formation.

2). LRP6 is involved in Wnt signal reception

LRP6 may activate Wnt signal transduction by functioning in the reception of the Wnt signal in responding cells, or by enhancing Wnt ligand generation or secretion in Wnt producing cells. To distinguish between these two possibilities, Wnt-5a and LRP6 were injected separately into neighboring blastomeres at the 4-cell stage (Fig. 3a, insert). Induction of secondary axes in embryos as well as Xnr3 and sia expression in explants was observed even when Wnt-5a and LRP6 were expressed in different cells in the embryo (Fig. 3a and b). Therefore, LRP6 function in Wnt signaling is likely involved in the reception, rather than the production or secretion, of the Wnt ligand.

3). A dominant negative mutant LRP6 blocks Wnt signaling

In an attempt to interfere with the function of the endogenous Xenopus LRP6, which is expressed maternally and throughout embryogenesis (Fig. 4e), we generated LRP6ΔC that had most of its cytoplasmic domain deleted. LRP6ΔC completely lacked the ability, either alone or in combination with Wnt-5a, to induce axis duplication or to activate Xnr3 and sia expression (Fig. 4a and b). Moreover, LRP6ΔC inhibited axis duplication and Xnr3/sia gene induction by the wild type LRP6 (Fig. 4a and b). This inhibition was counteracted by an increasing amount of co-injected LRP6 RNA (Fig. 4a). These results suggest that LRP6ΔC is a dominant-negative interfering mutant for LRP6 or related molecules, and that LRP6 cytoplasmic domain is required for Wnt signaling. LRP6ΔC

inhibited Xnr3 and sia induction by several Wnt molecules, including Wnt-1, Wnt-2, Wnt-3a and Wnt-8 (Fig. 4c), suggesting that LRP6 or a related molecule is required for signaling by these Wnt ligands. LRP6ΔC also inhibited Wnt-5a signaling via hFz5 (Fig. 4c), demonstrating that hFz5, and most likely other endogenous Fz molecules mediating Wnt-1 or Wnt-8 signaling, depend on the function of LRP6 or related proteins. LRP6ΔC did not affect Xbra induction by either activin or bFGF (Fig. 4d), indicating that LRP6ΔC interfered specifically with Wnt signaling without affecting other transmembrane signaling pathways.

While LRP6ΔC blocked signaling by Wnt ligands, it did not perturb the endogenous axis formation upon dorsal injection at the 4-cell stage (data not shown). This was consistent with a possibility that the dorsal β-catenin pathway is activated by mechanisms other than Wnt stimulation (reviewed in 17); alternatively, the endogenous dorsal Wnt/Fz signaling may occur early (23) before LRP6ΔC could interfere. Nonetheless, LRP6ΔC inhibited neural crest development as assayed by slug expression, and suppressed ectopic neural crest formation induced by Wnt-3a plasmid (Fig. 2a and b). Further, co-injection of LRP6 RNA rescued the inhibition of neural crest formation by LRP6ΔC (Fig. 2b). These results demonstrate that LRP6 or a related molecule is required for Wnt-dependent neural crest formation in vivo.

Conclusion

We have demonstrated that in two Wnt pathway-dependent developmental processes in Xenopus, secondary axis and neural crest formation, expression of LRP6 activates whereas a dominant negative LRP6 mutant blocks Wnt signaling. These results provide compelling evidence that LRP6 plays a critical role in Wnt signal transduction. The pivotal role of LRP6 in Wnt signaling in Xenopus mirrors the requirement of *arrow* function in Wg signaling in Drosophila (11), and is consistent with the observation that a lrp6 mutation in mice results in phenotypes recapitulating aspects of loss-of-function mutations of several, but not all, wnt genes (16). Thus, the LRP6/Arrow function in Wnt signal transduction is highly conserved. We are currently examine the molecular mechanism of LRP6 function in Wnt signal transduction. Given that LRP6 is a transmembrane receptor-like protein, we are particularly interested in whether it is a co-receptor molecule for Wnt molecules including Wnt-1.

Figure legends

Figure 1. Function of LRP6/LRP5 in Wnt signal transduction. (**a**) Ventral injection of RNA for LRP6 alone (500 pg or 2 ng), or co-injection of RNAs for Xenopus Wnt-5a (20 pg) plus either LRP6 (100 pg) or LRP5 (500 pg) induced axis duplication. LDLR RNA (5 ng or 1 ng, data not shown) alone or co-injected with Wnt-5a did not induced axis duplication. n: numbers of embryos scored in 2 to 5 experiments. (**b**) LRP6 (2 ng) or Wnt-5a (20 pg) plus LRP6 (100 pg) induced trunk axis duplication lacking head and the notochord. Top: the whole embryo phenotype at stage 40. Bottom: histology on cross sections. Note the presence of muscle (M) and neural tissues (N), and the absence of the notochord (No) in the induced axis (axis 2). (**c**) Ventral co-injection of Wnt-5a (20 pg) plus LRP6 (100 pg) activated ectopic Xnr3 expression at stage 10.5, as assayed by whole-mount in situ hybridization. Wnt-5a/LRP6 induced Xnr3 expression was weaker than the endogenous Xnr3 expression. Note that neither Wnt-5a nor LRP6 alone induced Xnr3. 10 to 12 embryos were examined for each injection, and 100% embryos exhibited patterns shown. (**d, e**) Synergistic induction of Xnr3 and sia expression in animal pole explants at stage 10.5 by LRP6 (100 pg) plus Wnt-5a (20 pg) or hFz5 (400 pg), as assayed by RT-PCR. A synergy between Wnt-5a and hFz5 (ref. 4) was demonstrated for comparison. The RNA amount injected is as in (**a**) except for Xenopus Wnt-8 (10 pg). Note that Xbra was not induced. EF-1α was used as a loading control for RT-PCR. WE: whole embryos (stage 10.5) used as a positive control for RT-PCR; Con: explants from uninjected embryos.

Figure 2. Function of LRP6 in neural crest formation. (**a**) slug expression examined by whole mount in situ hybridization at stage 15 to 20. LRP6 RNA (2 ng) or Xenopus Wnt-3a plasmid (100 pg) increased, whereas LRP6ΔC (2 ng) inhibited slug expression in the injected half of the embryo. LRP6ΔC also antagonized Wnt-3a induction of slug expression, and inhibited the expression of sox9, another crest-specific transcription factor (not shown). Expansion of slug expression was mostly restricted to the anterior region of the injected half (the left side of embryos shown), which was labeled by β-gal staining (red) derived from co-injected β-galatosidase RNA (1 ng). LDLR (2 ng) did not affect slug expression. Note that Wnt-3a expression plasmid was injected, since zygotic Wnt signaling is involved in neural crest development. (**b**) Summary of the in situ hybridization results. n, total number of embryos examined in 2 to 6 independent experiments.

Figure 3. LRP6 mediates signaling by Wnt-5a produced in neighboring cells. Xenopus Wnt-5a RNA (100 pg) and LRP6 RNA (100 pg) were separately injected into different neighboring blastomeres in the ventral equatorial region (**a**) or in the animal pole (**b**). sep: separate injection. co: Wnt-5a plus LRP6 co-injected into a single blastomere for comparison. (**a**) Percentage of embryos with axis duplication. (**b**) Induction of Xnr3 and sia in animal pole explants. Compared with co-injections, separate injections yielded a lower percentage of axis duplication (**a**) and weaker induction of Xnr3 and sia (**b**), probably due to poor diffusion of secreted Wnt-5a protein.

Figure 4. LRP6 function is required for Wnt signaling. (**a**) LRP6ΔC (2 ng, labeled as ΔC) did not induce axis duplication but inhibited axis induction by LRP6 (500 pg) when co-injected. An increase in LRP6 RNA amount (1 ng) counteracted the inhibition by LRP6ΔC. (**b** to **d**) Animal pole explant assays. (**b**) LRP6ΔC alone (2 ng) or Wnt-5a (20 pg) plus LRP6ΔC (100 pg) did not induce Xnr3 and sia expression. LRP6ΔC (500 pg to 2 ng) inhibited Xnr3 and sia expression induced by Wnt-5a (20 pg) plus LRP6 (100 pg) in a dose dependent manner. (**c**) LRP6ΔC (2 ng) inhibited Xnr3 and sia induction by Wnt-1 (10 pg), Wnt-2 (40 pg), Wnt-3a (5 pg), Wnt-8 (10 pg), and by Wnt-5a (20 pg) plus hFz5 (100 pg). Xenopus Wnts were used except for mouse Wnt-1. (**d**) LRP6ΔC (2 ng) did not perturb Xbra induction by activin (50 ng/ml) or bFGF (50 ng/ml). (**e**) Xenopus LRP6 is expressed maternally and throughout embryogenesis as assayed by RT-PCR. ODC was used as a control for relative RNA amounts. Genomic contamination was ruled out by PCR without RT (-RT).

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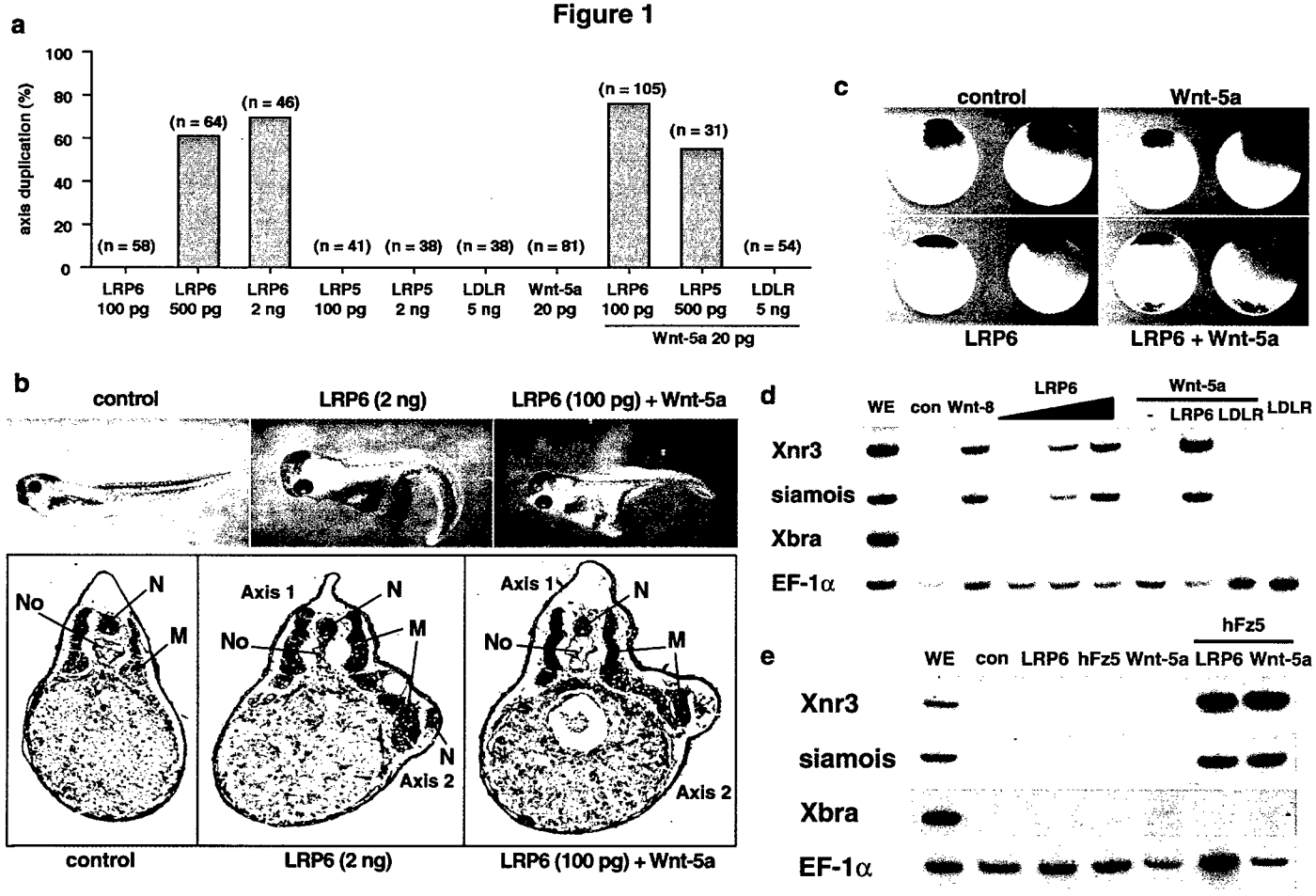
Figure 1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control
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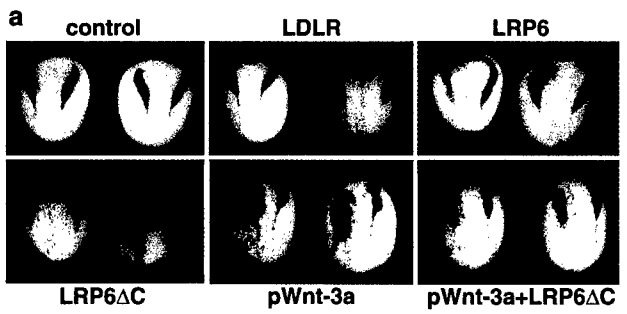
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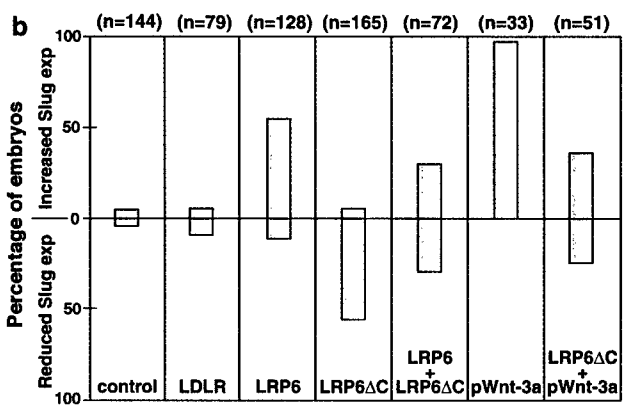
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Reduced Slug exp
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50
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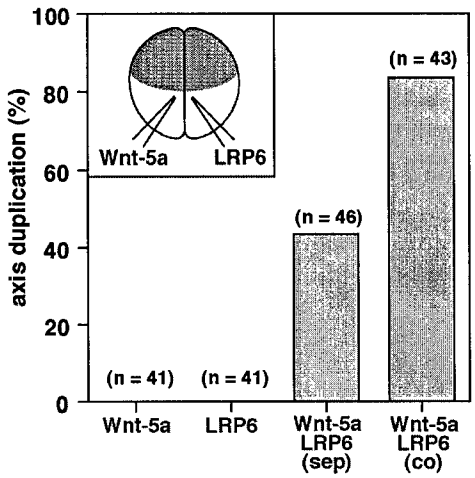
LRP6ΔC + pWnt-3a

Figure 3

a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b

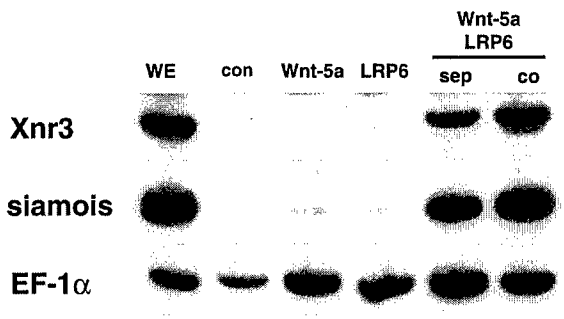
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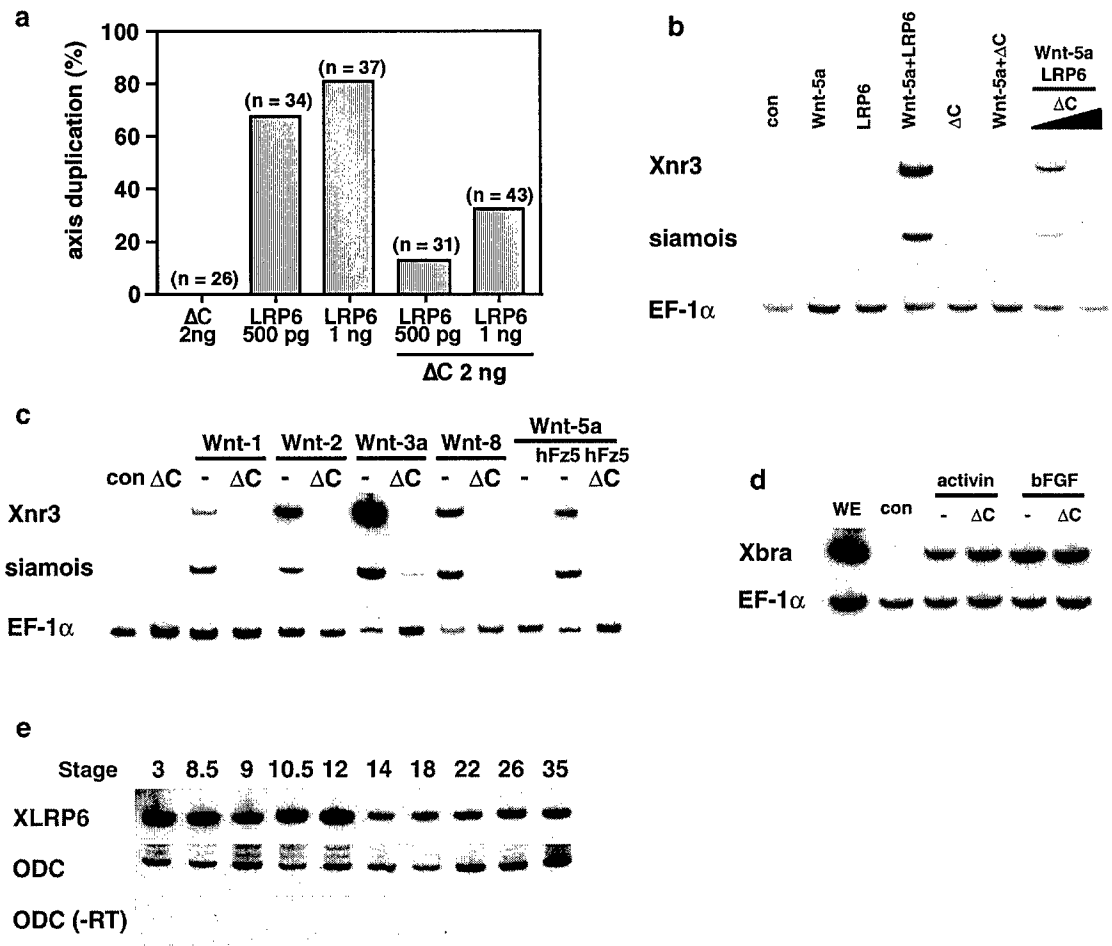
Figure 4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bFGF
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ODC (-RT)